Glycosylation by Chinese hamster ovary cells in dolichol phosphate-supplemented cultures

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N-linked glycosylation often imparts important properties to protein therapeutics. An essential step in this intracellular process is the transfer of oligosaccharide from dolichol monophosphate (Dol-P) to a potential glycosylation site. Variability in the success rate of this reaction affects the extent of protein glycosylation. The critical role of Dol-P suggests that its availability may influence the extent of glycosylation by limiting the pool of lipid-linked oligosaccharides (LLOs), the glycosyl donor. To test this hypothesis, the impact of Dol-P supplementation on protein glycosylation in Chinese hamster ovary (CHO) cells was investigated. Although exogenous Dol-P was incorporated by CHO cells and processed into LLOs in a dose-dependent manner, Dol-P supplementation had no marked effects on LLO or overall cellular glycosylation levels. While concentrations of exogenous Dol-P exceeding 100 μg/ml were detrimental to CHO cell viability, maximum non-toxic supplemental doses of Dol-P had no significant impact on the glycosylation of recombinant interferon-γ produced by batch cultures of CHO cells. These results show that glycosylation in CHO cells cannot be readily enhanced by Dol-P feeding under normal culture conditions.

Introduction

N-linked glycosylation is of particular interest to the biopharmaceutical industry because it can influence the blood plasma clearance rate, antigenicity and other important biological activities of glycoprotein therapeutics [1].

An essential step in N-linked glycosylation is the *en bloc* transfer of an oligosacccharide from dolichol monophosphate (Dol-P), a long-chain polyisoprenoid lipid in the endoplasmic reticulum (ER) membrane, to a potential glycosylation site on a nascent polypeptide [2]. The success rate of this glycosyl transfer reaction is usually variable, even among molecules of the same protein. Therefore, a given protein often emerges from the glycosylation pathway as a set of polypeptides differing in the number of attached oligosaccharide chains [3]. This variability in glycosylation is known as site occupancy heterogeneity.

Since proteins that differ in glycosylation can have different biological properties, heterogeneity in protein glycosylation presents special challenges to the development and production of a candidate glycoprotein therapeutic with consistent properties [4].

Information on the control of the glycosylation pathway remains inconclusive even though the individual glycosylation reactions and the function of Dol-P have been characterized in detail [2]. The critical role Dol-P plays as a glycosyl carrier suggests that the intracellular amount of Dol-P may determine the flux through the glycosylation pathway by controlling the availability of the carbohydrate donor in N-linked glycosylation, a lipid-linked oligosaccharide (LLO) moiety. Support for this hypothesis has come from different lines of investigation. Research on the developmental changes in oviducts of hormone-treated chicks [5] and embryos of sea urchins [6] demonstrated clear correlations between Dol-P levels and glycoprotein synthesis. Studies on the impact of exogenous Dol-P supplementation on mammalian cultures provided compelling evidence for the regulatory role of Dol-P in N-linked glycosylation: exogenous Dol-P elevated glycosylation levels in mammalian cultures by over 200% [7-10].

Although literature results indicate that cellular levels of Dol-P in mammalian cells are subsaturating, the ability of exogenous Dol-P to enhance glycosylation in Chinese hamster ovary (CHO) cells under normal culture conditions has not been elucidated. This work was conducted to determine whether glycosylation in typical CHO batch cultures is limited by Dol-P availability.

To test this hypothesis, the impact of exogenous Dol-P supplementation on a recombinant CHO cell line expressing human interferon-γ (IFN-γ) was studied. First, the uptake and incorporation of exogenous Dol-P into LLO by CHO cells was verified. Next, dosing studies were conducted to establish maximum non-toxic concentrations of exogenous

Dol-P for use in subsequent experiments. Third, the impact of Dol-P feeding on IFN-γ glycosylation was investigated. Finally, relative LLO and overall cellular glycosylation levels in CHO cells cultured in varying amounts of exogenous Dol-P were compared. Results here determine the applicability of using Dol-P supplementation to control glycosylation in CHO cells, and thereby guide the development of strategies for producing recombinant glycoproteins from CHO cultures with consistent glycosylation.

Materials and methods

CHO cell line and culture
The recombinant human IFN-γ-expressing CHO cell line used in all the experiments was obtained from Dr Walter Fiers at the University of Ghent, Ghent, Belgium [11] and has been described previously [12,13]. It was adapted to grow in suspension in CHO-S-SFM II [12], a commercially available serum-free medium (Gibco BRL). All experiments were conducted by culturing these cells in CHO-S-SFM II supplemented with 0.25 μM methotrexate, 10000 units/l penicillin and 10 mg/l streptomycin in shake flasks agitated at 70 rev./min in a 37 °C incubator with 5–10% CO_2 overlay and 95% humidity. For each separate experiment, a vial of frozen cells was taken from a working cell bank and inoculated in the serum-free medium. The cells were subsequently passaged 2–4 times and maintained at viabilities exceeding 95% until a sufficient number of cells was obtained to start the experiment. In this way, all cells used within an experiment had the same culture history.

Dol-P supplementation
Unlabelled Dol-P (Sigma) was originally dissolved in chloroform/methanol (2:1, v/v). The organic solvents were removed using a vacuum pump and the remaining yellow oil was redissolved in DMSO. Before placing this solution into the culture medium, serial dilution of Dol-P with DMSO was performed to obtain the required concentrations of Dol-P. [^{3}H]Dol-P (American Radiolabelled Chemicals) was purchased already dissolved in DMSO.

Dol-P supplementation was accomplished by vigorously mixing Dol-P (dissolved in DMSO) with culture medium in a shake flask. The Dol-P-supplemented flasks were then preincubated at 37 °C with agitation (70 rev./min) for 1 h prior to the introduction of CHO cells at the start of each experiment. The final DMSO concentration in all culture flasks was 0.5% (v/v). The actual concentration of supplemental Dol-P in the culture medium depended on the specific experiment that was performed.

Analytical methods
Cells were counted with a haemacytometer and microscope. Cell viabilities were determined by Trypan Blue dye ex-

clusion. The mode of cell death was studied by morphological examination of cells stained with Acridine Orange/ethidium bromide (AO/EB) dye [14]. Cell-cycle distributions were determined by flow cytometry of propidium iodide-stained cells [15]. Analyses of IFN-γ glycosylation site occupancy [13] and glycosylation microheterogeneity [12] were described previously in detail. Concentrations of IFN-γ were measured with an ELISA kit (Biosource International). Glucose and lactate concentrations in the culture media were measured directly by the YSI 23 000 STAT Plus Glucose & Lactate Analyser (YSI Life Sciences).

LLOs and cellular protein extractions
Between 5×10^6 and 10×10^6 cells were taken from each culture for extraction of LLOs and cell-associated proteins based on a well-established sequential extraction procedure [16–18]. Briefly, the cell pellets were delipidated by thorough mixing with chloroform/methanol (2:1, v/v). After washing with water, LLOs were extracted with chloroform/methanol/water (10:10:3, by vol.). The residue remaining after lipid extraction contained cellular proteins. After precipitating the proteins with ice-cold 10% (w/v) tricholoracetic acid, 0.5 ml of warm 1 M NaOH was added to dissolve the proteins. When cells are treated in this manner the combined chloroform/methanol/water (10:10:3, by vol.) extracts can be taken to consist primarily of LLOs [19–22], and the delipidated residue can be assumed to be comprised of proteins [16,18,19,23].

Radioactivity measurements
Radioactivity was measured in disintegrations/min (DPM) with an LS 6500 Liquid Scintillation Counter (Beckman Instruments). Organic solvents were removed from radiolabelled LLO extracts by drying in a fume hood prior to liquid scintillation counting. Unless otherwise stated, 20 ml of the scintillation cocktail Ultima Gold (Packard Bioscience) was used to dissolve the radiolabelled samples.

Uptake of exogenous Dol-P
Prior to starting this time-course experiment, labelled and unlabelled forms of Dol-P dissolved in DMSO were mixed such that 0.4% of the total Dol-P was tritium-labelled. In this experiment, four CHO batch cultures were run simultaneously. The first flask was the control while the other three flasks contained increasing concentrations of Dol-P. At the start of the experiment CHO cells growing exponentially in a single flask of CHO-S-SFM II medium were divided into four centrifuge tubes, spun down and resuspended at approx. 3.8×10^5 cells/ml into each of the four separate flasks. Then, 1 h into the experiment, three samples (50 μl each) were drawn at random from each culture flask and assayed for [^{3}H]Dol-P radioactivity to be certain of an even distribution of the exogenous lipid in the cultures, and to verify the actual

amount of [³H]Dol-P supplied to the medium. Subsequently, samples were taken from each flask at 24 h intervals for cell counting and LLO extraction.

Toxicity studies on high doses of supplemental Dol-P
Suspended CHO cells were seeded at 3.5×10^5 cells/ml into three different flasks containing 0 (control), 120 and 250 µg/ml Dol-P. Samples were taken from the flasks daily for (i) cell counting, (ii) determination of cell viability and mode of cell death and (iii) cell-cycle analysis.

Impact of Dol-P on IFN-γ glycosylation
Exponentially dividing CHO cells were divided into four flasks (each containing 3.0×10^5 cells/ml) for radiolabelling studies. One flask served as the control and the three test flasks contained different concentrations of Dol-P (60, 100 and 180 µg/ml). Culture supernatant collected at the end of the experiment was filtered and frozen at −20 °C until it was thawed for IFN-γ glycosylation and concentration analyses.

Relative LLO and glycosylation levels in Dol-P-supplemented cells
Two sets of experiments employing different radiolabels were performed. In the first experiment, fresh labelling medium comprised of CHO-S-SFM II supplemented with 8.4 µCi/mld-[U-¹⁴C]glucose (American Radiolabelled Chemicals) and 3.3 µCi/ml L-[³H]leucine (Amersham Biosciences) was transferred into four identical shake flasks. The first flask served as a control, and the other three flasks were supplemented with increasing amounts of unlabelled Dol-P to give final concentrations of 20, 50 and 100 µg/ml Dol-P, respectively. To start the experiment, CHO cells that had been growing exponentially for 22 h in a shake flask of labelling medium were divided into three centrifuge tubes and collected by centrifugation (5 min, 200 g). Each cell pellet was resuspended at $(3–4) \times 10^5$ cells/ml in fresh labelling medium into one of the four flasks. At regular time intervals, culture samples were taken for various measurements. The cells were separated from the culture supernatant and used for cell enumeration and extraction of LLOs and cellular proteins. The supernatant was filtered and stored at −20 °C until it was used for metabolite analyses.

In the second experiment, fresh CHO-S-SFM II medium supplemented with 48 µCi/ml D-[2-³H]mannose (American Radiolabelled Chemicals) and 0.3 µCi/ml L-[³⁵S]methionine (Amersham Biosciences) was divided into three identical shake flasks. Unlabelled Dol-P of different concentrations was then added such that the flasks contained 0, 20 and 50 µg/ml Dol-P, respectively. After 1 h of preincubation

with agitation, CHO cells that were exponentially growing in unlabelled media were introduced into the three shake flasks at $(3–4) \times 10^5$ cells/ml to start the experiment. Subsequently, at regular time intervals, LLOs and cellular proteins were isolated from the three parallel batch cultures using the methods described above for radioactivity measurements.

Results and discussion

Uptake of exogenous Dol-P
The uptake of exogenous Dol-P has been demonstrated in several types of mammalian cell and tissue, but not in CHO cells [7–10]. This experiment was conducted to determine the ability of CHO cells to incorporate exogenous Dol-P supplied to the cultures in the same manner (that is, dissolved in DMSO) [7–10]. To this end, unlabelled Dol-P was spiked with the radioactive tracer [³H]Dol-P.

To ascertain the even distribution of supplemental Dol-P throughout the aqueous culture medium, three samples were drawn at random from each culture flask and assayed for [³H]Dol-P radioactivity 1 h after starting the experiment. The random samplings confirmed that the exogenous lipid was well dispersed in the cultures; [³H]Dol-P radioactivity measured in the three samples from each flask varied by less than 10% (results not shown). The amount of Dol-P present in each flask was calculated from their [³H]Dol-P radioactivities based on the fact that 0.4% of the supplemental lipid in the medium was radiolabelled. Radioactivity measurements of the culture flasks indicated the presence of 0, 0.59, 0.85 and 1.74 µCi/ml [³H]Dol-P, which corresponded to 0, 12, 17 and 35 µg/ml Dol-P, respectively.

Radioactivities in LLOs extracted from the cells throughout the course of culture increased in proportion with the exogenous concentration of supplemental Dol-P (Figure 1). Extraction of CHO cells with chloroform/
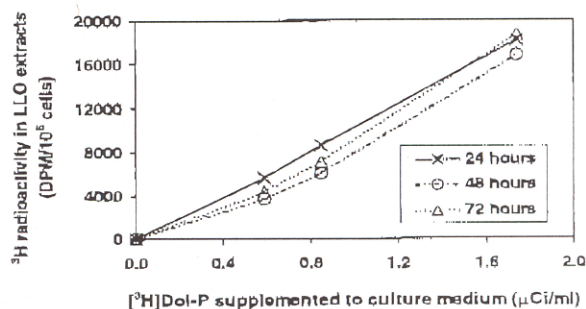

Figure 1 Incorporation of exogenous Dol-P into LLO by CHO cells

Batch cultures of CHO cells in serum-free medium supplemented with various amounts of exogenous Dol-P were run simultaneously. The concentrations of supplemental [³H]Dol-P in the four cultures were 0, 0.59, 0.85 and 1.74 µCi/ml, respectively, as determined by radioactivity measurements.
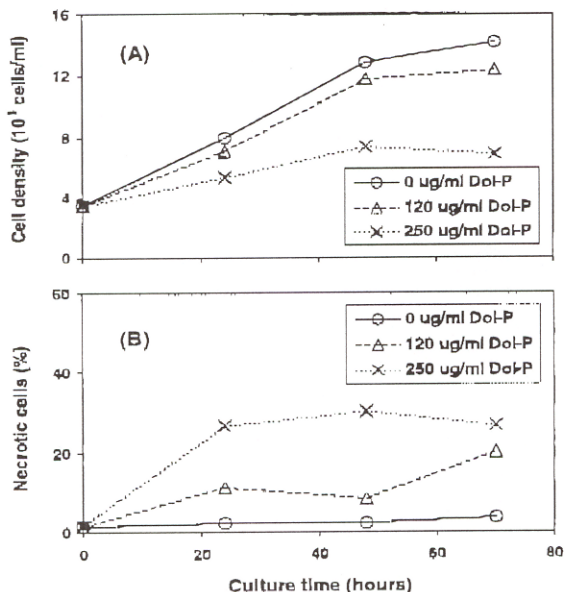
Figure 2 Cell density (A) and percentage of necrotic cells (B) in CHO batch cultures supplemented with high concentrations of Dol-P

Cultures containing 0 (O), 120 (△) or 250 μg/ml (×) Dol-P were run simultaneously. The distribution of necrotic cells in cultures was determined by AO/EB assay.

Table 1 Impact of Dol-P supplementation on recombinant IFN-γ glycosylation site occupancy and specific productivity

CHO cells were cultured in the presence of 0, 60, 100 or 180 μg/ml supplemental Dol-P. Glycosylation site occupancy of IFN-γ accumulated in medium after 62 h of batch culture was analysed by capillary electrophoresis. Three forms of recombinant human IFN-γ were secreted by the CHO cells: two sites glycosylated, one site glycosylated and no sites glycoslated. Specific IFN-γ productivity (q_{IFN}) was calculated by dividing the IFN-γ concentration in the medium after 62 h of culture by the integrated viable cell density.

| | Percentage of secreted IFN-γ | | | |
Supplemental Dol-P	Two sites glycosylated	One site glycosylated	No sites glycosylated	q_{IFN} (pg/cell per day)
0 μg/ml	67.3	25.5	7.2	2.1
60 μg/ml	70.5	25.5	4.0	2.1
100 μg/ml	73.5	19.0	7.5	1.8
180 μg/ml	64.8	27.3	7.9	1.5

methanol/water (10:10:3, by vol.) has been shown to yield primarily LLOs [24,25], which are formed in the ER by the sequential addition of monosaccharides to Dol-P [2]. Hence the findings here show that CHO cells transported exogenous Dol-P to the ER and used it for LLO biosynthesis.

Toxicity studies on high doses of supplemental Dol-P
Incubations of mammalian cells and tissues with exogenous Dol-P typically used less than 20 μg/ml Dol-P [7,8,10]. The highest concentration of exogenous Dol-P recorded for *in vivo* studies was 100 μg/ml Dol-P [9]. The range of Dol-P concentrations used in the previous experiment (12–35 μg/ml Dol-P) had negligible impact on CHO cell growth and viability (results not shown).

To determine the upper limit of supplemental Dol-P concentration for use in subsequent experiments, CHO cells were cultured in the presence of large doses of exogenous Dol-P. At the highest Dol-P concentration (250 μg/ml), cell densities were significantly lower than in the other cultures (Figure 2A). This detrimental effect became increasingly apparent as cultivation time progressed.

Cultured cells can die from necrosis or apoptosis [14]. Necrosis occurs in response to physical or chemical trauma to the cell, and it leads to disruption of the cell membrane. Unlike necrosis, apoptosis is regulated by events within the

cell. Cells with higher plasma membrane fluidity have increased shear sensitivities [26]. Since Dol-P destabilizes phospholipid bilayers [27,28], cell membranes with a higher Dol-P content would be more susceptible to shear-induced necrotic death. Consistent with this hypothesis, AO/EB assays show that the culture with the highest concentration of supplemental Dol-P (250 μg/ml) consistently contained the largest proportion of necrotic cells (Figure 2B). Compared with the control culture, the fraction of necrotic cells in the 120 μg/ml Dol-P-supplemented culture was noticeably higher only after 70 h of culture. At the end of the experiment, the percentage of cells undergoing necrosis was 4% in the control culture, 20% in the 120 μg/ml Dol-P culture and 27% in the 250 μg/ml Dol-P culture.

Large doses of Dol-P did not have a noticeable effect on cell-cycle distributions (results not shown), suggesting that the negative impact of large quantities of exogenous Dol-P on cell growth resulted from the increase in necrotic cell death rather than growth arrest.

Impact of Dol-P on IFN-γ glycosylation
The impact of exogenous Dol-P on the glycosylation of a specific recombinant protein has not been recorded previously. In this experiment, CHO cells were grown in the presence of 60, 100 or 180 μg/ml supplemental Dol-P to determine the maximum possible effect of Dol-P feeding on IFN-γ glycosylation.

Even at the large supplemental doses of Dol-P used in this study, no significant improvement in IFN-γ glycosylation site occupancy was observed (Table 1). Compared with the control sample, the percentage of doubly glycosylated IFN-γ was less than 10% higher in the 100 μg/ml Dol-P sample, and it was slightly lower in the 180 μg/ml Dol-P sample. The poorer glycosylation at this Dol-P concentration is attributed to the low culture viability; less than 50% of the cells in the
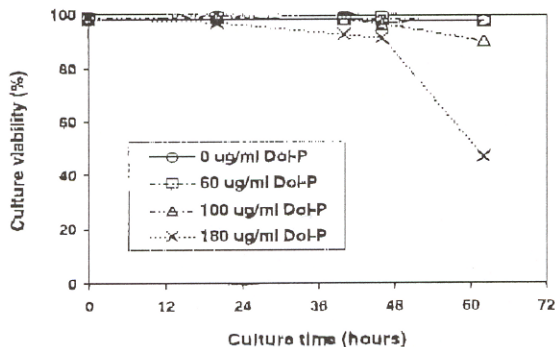
Figure 3 Impact of Dol-P supplementation on culture viability

CHO cells were grown in the presence of 0, 60, 100 or 180 μg/ml supplemental Dol-P. Culture viabilities were determined by Trypan Blue dye exclusion assay.
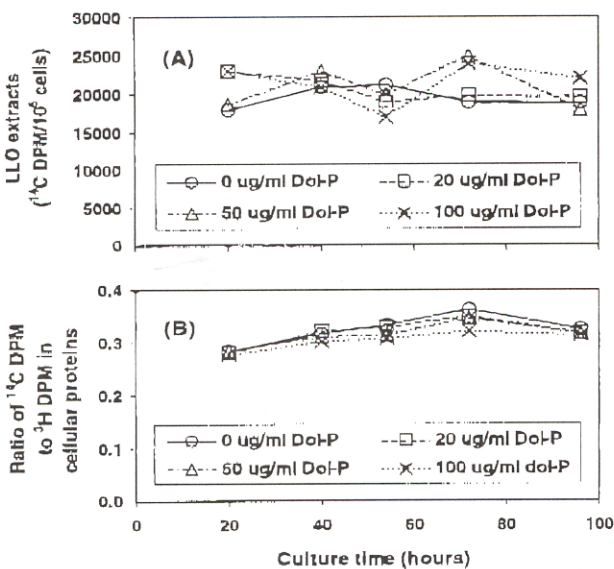

Figure 4 Relative LLO and glycosylation levels in CHO cells cultured in the presence of [14C]glucose and [3H]leucine as well as various amounts of exogenous Dol-P

Relative LLO levels were represented by 14C radioactivity measured in LLO extracts were normalized to cell number (A). Relative glycosylation levels were indicated by the ratio of 14C to 3H radioactivities measured in CHO cellular proteins (B). All cultures contained [14C]glucose and [3H]leucine as well as supplemental Dol-P at 0, 20, 50 or 100 μg/ml. LLOs and cellular proteins were extracted from cells growing in the four parallel CHO batch cultures at regular time intervals.

180 μg/ml Dol-P culture were viable after 62 h of culture (Figure 3).

Consistent with the fact that Dol-P is not involved in the subsequent branching and sugar trimming, and addition reactions that lead to glycosylation microheterogeneity, Dol-P supplementation had no obvious impact on the sialylation of IFN-γ biantennary glycans or on the branching

pattern (antennarity) of IFN-γ glycans at both glycosylation sites (results not shown).

Although inhibition of Dol-P synthesis can hinder glycoprotein production and cell growth [29], an increased availability of Dol-P under normal culture conditions does not necessarily generate the opposite effect. Cultures supplemented with Dol-P did not show higher specific IFN-γ productivities (Table 1) or cell densities (results not shown). These results indicate that Dol-P availability does not limit cell growth or IFN-γ production and glycosylation in these recombinant CHO cells under normal culture conditions.

Relative LLO and glycosylation levels in Dol-P supplemented cells

Dol-P supplementation has been shown to significantly enhance LLO and overall glycosylation levels in non-CHO mammalian systems. Kousvelari and co-workers [9] showed that incorporation of radiolabelled mannose into total trichloroacetic acid-precipitated glycoproteins in dispersed rat parotid gland acinar cells was increased 3-fold by 10 μg/ml Dol-P, and 5-fold at the maximum Dol-P concentration of 100 μg/ml in the culture medium. Supplementation of Madin-Darby canine kidney cell cultures with exogenous Dol-P stimulated mannose incorporation into both LLOs and glycoproteins by over 150% at 2.5 μg/ml and 250% at 10 μg/ml [10]. Preincubation of mouse LM cells with Dol-P (5 μg/ml) resulted in a 3-fold stimulation of LLO synthesis [8]. These findings support the hypothesis that subsaturating pools of endogenous Dol-P limit protein glycosylation in mammalian cells by constraining LLO levels.

To test the applicability of this hypothesis to the CHO glycosylation system, CHO cells were cultured in the presence of unlabelled Dol-P. The previous experiment demonstrated that Dol-P supplementation had minimal impact on IFN-γ glycosylation, but it did not show the effects on the majority of CHO proteins. These two experiments were conducted to examine the impact of exogenous Dol-P on relative LLO levels and on the overall extent of cellular protein glycosylation in CHO cells. These two experiments differed mainly in the set of radioisotopes used to label the LLOs and proteins: in the first experiment [14C]glucose and [3H]leucine were used, while the corresponding set of radiolabels used in the second experiment was [2-3H]mannose and [35S]methionine. To maximize any positive impact Dol-P feeding may have on glycosylation as well as to simultaneously avoid the adverse effects of large doses of exogenous Dol-P on cell viability, at least 50 μg/ml Dol-P was used in both experiments. Cellular incorporation of supplemental radiolabelled monosaccharides and amino acids were monitored over the course of culture to determine the impact of Dol-P supplementation on the
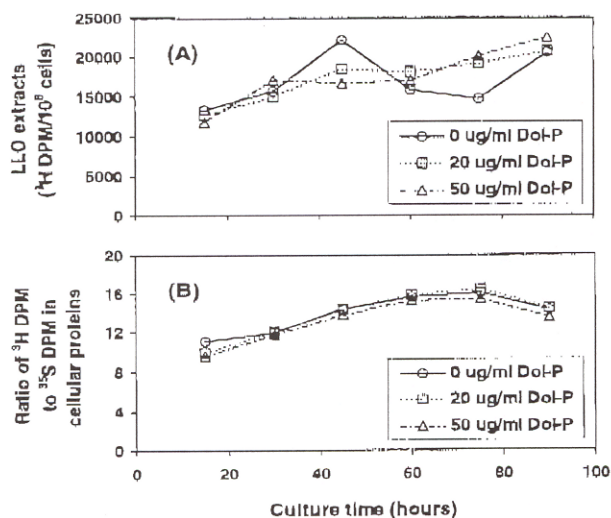
Figure 5 Relative LLO and glycosylation levels in CHO cells cultured in the presence of [2-³H]mannose and [³⁵S]methionine as well as various amounts of exogenous Dol-P

Relative LLO levels were represented by ³H radioactivity measured in LLO extracts normalized to cell number (A), and relative glycosylation levels were indicated by the ratio of ³H to ³⁵S radioactivities measured in cell-associated proteins (B). CHO batch cultures containing 0, 20 and 50 μg/ml Dol-P respectively were run simultaneously in the presence of [2-³H]mannose and [³⁵S]methionine. LLOs and cellular proteins were extracted from the three parallel CHO batch cultures at regular time intervals and assayed for incorporation of radioactivity.

relative levels of LLO and protein glycosylation. Radioactivity measured in LLO extracts originating from the radiolabled monosaccharide would indicate the relative amount of LLOs, while the ratio of sugar-derived to amino acid-derived radioactivities detected in cell-associated proteins would represent the relative extent of cellular glycosylation.

Despite the relatively large amounts of Dol-P used (20 and 50 μg/ml in both experiments, and an additional 100 μg/ml in the first experiment), Dol-P supplementation had no clear impact on LLO or glycosylation levels in CHO cells (Figures 4 and 5). In both sets of experiments, parallel cultures demonstrated identical glucose and lactate concentrations in the medium over time (results not shown). By contrast, Dol-P supplementations at 20 μg/ml or lower were able to elevate glycosylation levels in other mammalian systems by over 200% [7–10]. Although Dol-P supplementation to CHO cell cultures clearly did not lead to the multiple-fold stimulation of LLO and glycoprotein biosynthesis observed in other mammalian systems, the possibility that smaller changes in LLO levels resulting from Dol-P feeding cannot be excluded.

A major locus of control for N-linked glycosylation has been postulated to be the level of Dol-P in ER membranes; subsaturating levels of Dol-P can limit LLO availability and consequently the cellular glycosylation capacity. Despite the wealth of evidence supporting the regulatory role of Dol-P in mammalian glycosylation [5–10], the availability of endogenous Dol-P is not always limiting. Studies in mouse L-1210 cells showed that glycoprotein synthesis remained constant despite 2-fold variations in Dol-P levels during the cell cycle [30]. When calf thyroid microsomes were given Dol-P directly, or agents that allowed Dol-P to recycle, the LLO levels doubled, but protein glycosylation remained unchanged [21].

This study using CHO cells concurs with the findings in mouse L-1210 cells [30] and calf thyroid microsomes [21]. Results here demonstrate conclusively that the ability of exogenous Dol-P supplementation to stimulate glycosylation is not universal for all mammalian systems, and should be examined on a case-by-case basis.

Acknowledgments

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